JOURNAL OF BACTERIOLOGY, Nov. 2010, p. 5559–5571 Vol. 192, No. 21
0021-9193/10/$12.00 doi:10.1128/JB.00464-10

Mechanism of Positive Regulation by DsrA and RprA Small Noncoding RNAs: Pairing Increases Translation and Protects *rpoS* mRNA from Degradation▽

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Received 22 April 2010/Accepted 17 August 2010

Small noncoding RNAs (sRNAs) regulate gene expression in *Escherichia coli* by base pairing with mRNAs and modulating translation and mRNA stability. The sRNAs DsrA and RprA stimulate the translation of the stress response transcription factor RpoS by base pairing with the 5′ untranslated region of the *rpoS* mRNA. In the present study, we found that the *rpoS* mRNA was unstable in the absence of DsrA and RprA and that expression of these sRNAs increased both the accumulation and the half-life of the *rpoS* mRNA. Mutations in *dsrA*, *rprA*, or *rpoS* that disrupt the predicted pairing sequences and reduce translation of RpoS also destabilize the *rpoS* mRNA. We found that the *rpoS* mRNA accumulates in an RNase E mutant strain in the absence of sRNA expression and, therefore, is degraded by an RNase E-mediated mechanism. DsrA expression is required, however, for maximal translation even when *rpoS* mRNA is abundant. This suggests that DsrA protects *rpoS* mRNA from degradation by RNase E and that DsrA has a further activity in stimulating RpoS protein synthesis. *rpoS* mRNA is subject to degradation by an additional pathway, mediated by RNase III, which, in contrast to the RNase E-mediated pathway, occurs in the presence and absence of DsrA or RprA. *rpoS* mRNA and RpoS protein levels are increased in an RNase III mutant strain with or without the sRNAs, suggesting that the role of RNase III in this context is to reduce the translation of RpoS even when the sRNAs are acting to stimulate translation.

RpoS is a general stress response sigma factor made in *Escherichia coli* in response to several types of stresses and in the stationary phase of growth. An increase in the cellular levels of RpoS results in transcription of many genes, whose products help the cell cope with stress. The levels of RpoS in the cell are very tightly controlled and are modulated in response to specific changes in the growth environment (reviewed in reference 19).

Regulation of RpoS levels in response to environmental signals occurs at the levels of transcription, translation, and protein stability. In the absence of stress, when cells are growing rapidly, RpoS levels are very low, synthesis is low, and the protein is rapidly degraded. When cells run out of nutrients or encounter other types of stress, RpoS levels rise rapidly, a result of changes in both synthesis and degradation.

For instance, during exponential growth at 37°C, RpoS is rapidly degraded by the ClpXP ATP-dependent protease (46). This degradation requires the adaptor protein RssB (also called SprE), which binds to RpoS and delivers it to ClpXP (37, 40, 64). In response to specific suboptimal growth conditions, RpoS becomes stable. Stabilization of RpoS occurs in response to specific antiadaptor proteins that bind to RssB, blocking its ability to deliver RpoS to the protease. Antiadaptors made in response to phosphate starvation, magnesium starvation, and DNA damage have been described (3, 4).

The major translational control of RpoS depends upon the 5′ untranslated region (UTR) of *rpoS* and small noncoding RNAs (sRNAs). This regulation was first demonstrated in experiments showing that translation of *rpoS* is enhanced during growth at low temperature (20 to 25°C). Translational control under this growth condition is completely dependent on the presence of the sRNA DsrA (48). Transcription of DsrA is activated at low temperature, and upon accumulation, this sRNA base pairs with *rpoS* mRNA in the 5′ UTR, resulting in increased translation of RpoS (28, 43; reviewed in reference 30).

Regulation of translation by sRNAs is a widely studied phenomenon in *E. coli* and is rapidly being recognized as an important mechanism in other bacteria (62). A major class of regulatory sRNAs is synthesized in response to many kinds of stresses and base pairs with target mRNAs. Base pairing is stimulated by the RNA chaperone Hfq, which binds to both the sRNA and the target mRNA (22; reviewed in reference 5). The most frequent outcome of pairing is negative regulation of the target mRNAs, including degradation of the mRNA. Degradation of those target mRNAs that have been tested in *E. coli* is mediated by the degradosome, a protein complex containing the endoribonuclease RNase E, the exoribonuclease polynucleotide phosphorylase (PNPase), and other components (16, 31, 35, 57).

A rarer outcome of pairing is positive regulation by sRNAs, such as has been seen for regulation of *rpoS* translation by DsrA (28). A second sRNA, RprA, stimulates RpoS synthesis in response to cell envelope stress and base pairs with *rpoS* mRNA in the same region of the 5′ UTR as DsrA (27, 29). A number of other examples of positive regulation by sRNAs have been described (34, 38, 41, 55, 60). Thus, it seems likely

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▽ Published ahead of print on 27 August 2010.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
CM1000	DJ480 Δ*dsrA14*	NM308 + P1(NM14)
CM1001	DJ480 Δ*dsrA14 rpoS**	NM307 + P1(NM14)
CM1010	DJ480 Δ*dsrA14 rne-3071 zce-726*::Tn*10*	CM1000 + P1 (EM1277)
CM1050	DJ480 Δ*dsrA14* Δ*rnc-1223*::*cat*	CM1000 + P1(CM1223)
CM1052	DJ480 Δ*dsrA14 rne-3071 zce-726*::Tn*10* Δ*rnc-1223*::*cat*	CM1010 + P1(CM1223)
CM1060	DJ480 Δ*dsrA14* Δ*ara714*	CM1000 + P1(LMG194), P1(EM1448)
CM1061	DJ480 Δ*dsrA14* Δ*ara714 rpoS**	CM1001 + P1(LMG194), P1(EM1448)
CM1062	DJ480 Δ*dsrA14 rprA1*::*kan* Δ*ara714*	CM1060 + P1(NM22521)
CM1063	DJ480 Δ*dsrA14 rprA1*::*kan rpoS** Δ*ara714*	CM1061 + P1(NM22521)
CM1064	DJ480 Δ*dsrA14 rne-3071 zce-726*::Tn*10* Δ*ara714*	CM1060 + P1(EM1277)
CM1080	DJ480 Δ*dsrA14* Δ*ara714* Δ*rnc-1223*::*cat*	CM1060 + P1(CM1223)
CM1082	DJ480 Δ*dsrA14 rprA1*::*kan* Δ*ara714* Δ*rnc-1223*::*cat*	CM1062 + P1(CM1223)
CM1083	DJ480 Δ*dsrA14 rprA1*::*kan rpoS** Δ*ara714* Δ*rnc-1223*::*cat*	CM1063 + P1(CM1223)
CM1086	DJ480 Δ*dsrA14 rne-3071 zce-726*::Tn*10* Δ*rnc-1223*::*cat* Δ*ara714*	CM1064 + P1(CM1223)
CM1223	NM300 Δ*rnc-1223*::*cat*	This study
DJ480	MG1655 Δ*lac*X74	7
DY330	W3110 Δ*lacU169 gal-490* λcI857 Δ(*cro-bioA*)	63
EM1277	DJ480 *rne-3071 zce-726*::Tn*10*	31
EM1448	DJ480 Δ*ara714 leu*$^+$	Lab strain
JNB001	DJ480 Δ*dsrA14 rprA1*::*kan*	CM1000 + P1(NM22521)
JNB002	DJ480 Δ*dsrA14 rprA1*::*kan rpoS**	CM1001 + P1(NM22521)
LMG194	Δ*ara714 leu*::Tn*10*	18
MG1655	Wild type	Lab strain
NC397	HME45 *lacI′<>kan-Ter<>cat-sacB<>lacZYA*	51
NM1	DY330 Δ*dsrA14*::*cat-sacB*	This study
NM14	DY330 Δ*dsrA14*	This study
NM22521	C600 *rprA1*::*kan*	Lab strain
NM300	DJ480 mini-λ *tet*	54
NM301	NM300 *rpoS**	This study
NM307	NM301 *rpoS** Δ*dsrA*::*cat-sacB his*::Tn*10*	NM301 + P1(NM311)
NM308	DJ480 Δ*dsrA*::*cat-sacB his*::Tn*10*	DJ480 + P1(NM311)
NM111	Hfr H Δ*dsrA*::*cat-sacB his*::Tn*10*	SG2204 +P1(NM1)
SG2204	Hfr H *his*::Tn*10*	56
Plasmids		
pACS21	pBR322 *rnc*$^+$	53
pNM12	pBAD24 derivative	28
pNM13	pBAD-*dsrA*$^+$	28
pNM33	pBAD-*dsrA**	28
pNM100	pBAD-*rprA*$^+$	29
pNM109	pBAD-*rprA**	29

that the positive regulation seen with DsrA and RprA is mimicked by many other sRNAs. Many of these positively acting sRNAs also act negatively on other mRNAs.

A number of studies have examined the interaction of DsrA and RprA and their targets *in vitro* and *in vivo*, and crucial base pairs for the action of DsrA and RprA have been identified (25, 26, 28, 30, 33, 50). Furthermore, genetic studies and *in vitro* analysis have suggested that the *rpoS* mRNA forms a hairpin that occludes the ribosome binding site in the 5′UTR if Hfq or the sRNAs are absent (6, 26). *In vitro*, base pairing with the sRNA can relieve this occlusion (26). Hfq binding to multiple sites on the *rpoS* mRNA significantly enhances binding by the sRNAs (49, 58). However, little is known about the fate of the mRNA upon binding to an sRNA *in vivo*.

The present study analyzes the fate of the *rpoS* mRNA in the presence and absence of the sRNAs DsrA and RprA *in vivo*. We find that *rpoS* mRNA is unstable and is degraded in a fashion that involves the two major endoribonucleases, RNase E and RNase III. Expression of either DsrA or RprA can stabilize the mRNA, and this requires base pairing; the stabilization overcomes degradation by RNase E but not by RNase III. Blocking degradation of the mRNA is, however, not sufficient to improve RpoS translation; pairing with the sRNA is still necessary to allow productive translation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains were grown on LB (Lennox broth; KD Medical) plates at 37°C with or without the following antibiotics, as appropriate: ampicillin (Ap; 50 μg/ml), kanamycin (Km; 25 μg/ml), chloramphenicol (Cm; 10 μg/ml), or tetracycline (Tc; 25 μg/ml). Strains containing the *rne-3071* allele were maintained at 30°C. All *E. coli* strains used are derivatives of DJ480 and are listed in Table 1. DJ480 is a Δ*lacX74* derivative of MG1655 (7). The primers and biotinylated oligonucleotide probes used in this study were purchased from Integrated DNA Technologies and can be found in Table 2.

The *rpoS** allele replaces 3 nucleotides (nt) of the *rpoS* 5′ UTR with 5 nt, generating an NcoI restriction site in the *rpoS* DNA sequence, and was previously used and referred to as NcoI′ (28, 29). *rpoS** was introduced directly into the chromosome by bacteriophage lambda red recombination (63) using the single-stranded mutagenic oligonucleotide RpoS-NcoI-dpndnt. The NM300 strain (DJ480 carrying a mini-bacteriophage λ [54]) was electroporated with 100 ng of the oligonucleotide, resuspended in LB medium, serially diluted, plated on

TABLE 2. Oligonucleotide primers and probe used in this study

Primer or probe	Sequence
Primers	
ΔdsrAcat-For	CGTTGAATGCACAATAAAAAAATCCCGACCCTGAGGGGGTCGGGATGAAAATGA GACGTTGATCGGCACGTA
ΔdsrAsacB-Rev	CGTTAATCATTCATATGGCGAATATTTTCTTGTCAGCGAAAAAAATTGCGATCAAA GGGAAACTGTCCATATG
MtntSeq_RpoS-FOR	ATAGCGACCATGGGTAGCACC
Rnccatfor	TCGTGTGCTGAATTGTTGACGCATTTATTTATTGGTATCGCAAAATGAGACGTTGA TCGGCACG
Rnccatrev	TCCGACGATGGCAATAAATCCGCAGTAACTTTTATCGATGCAACCAGCAATAGAC ATAAGCG
RpoS-NcoI-dpndnt	CACCGGAACCAGTTCAACACGCTTGCATTTTGAAATTCCATGGACAAGGGGAAAT CCGTAAACCCGCTGCGTTATTTGCC
RpoS_NcoI-REV	CTTCTTCGGCCGTTAACAGTGGTG
rpoSRACE235	CGGCCGTTAACAGTGGTGAAT
ΔdsrA replacement oligonucleotide	CGTTGAATGCACAATAAAAAAATCCCGACCCTGAGGGGGTCGGGATGCGCAATTT TTTTCGCTGACAAGAAAATATTCGCCATATGAATGATTAACG
Probe RpoS-N3	CAAATCGTTATCACTGGGTTCCTG

LB medium, and counted. The leftover electroporated cells were diluted the next day into 100 μl each in a 96-well microtiter plate to have about 6 to 10 bacteria per well, and the plate was incubated overnight at 37°C. One microliter from each well was then screened by PCR using the RpoS_NcoI-REV and MtntSeq_RpoS-FOR primers, whose last 3 nucleotides at the 3′ end match the mutated sequence and not the wild-type sequence. Three wells yielded a PCR product, and their contents were serially diluted and plated for single colonies. Thirty-two colonies from the 3 wells were screened further by PCR, and 8 were positive for the mutation. These were purified, their DNA was sequenced to confirm the mutation, and one isolate was saved as NM301.

The unmarked Δ*dsrA14* deletion was constructed using the ΔdsrAcat-For and ΔdsrAsacB-Rev primers to amplify the *cat-sacB* cassette from strain NC397 (51) by PCR. The product was introduced by bacteriophage λ red recombinase-mediated recombination (recombineering) into strain DY330, selecting for Cm^r and screening for Suc^s, as described previously (63), to yield strain NM1. Recombineering was used again to replace the cassette in NM1 by the single-stranded Δ*dsrA* replacement oligonucleotide to yield strain NM14.

Transductions with $P1_{vir}$ were performed as described previously (47) to create the strains used here, as indicated in Table 1, generally selecting for antibiotic resistance. To make an unmarked deletion of *dsrA*, the Δ*dsrA*::*cat-sacB* allele from NM1 was first introduced into SG2204 to link the mutation to a *his*::Tn*10* auxotrophy marker (linkage, ~1%) to yield strain NM111. The linked *his* and *dsrA* mutations were then introduced into DJ480 and NM301 (*rpoS**), selecting for Tc^r and screening for Cm^r (linkage, ~1%), followed by P1 transduction from NM14, containing the Δ*dsrA14* allele, selecting for his^+ growth on M63 glucose minimal plates (KD Medical), and screening for Cm^s to yield strains CM1000 ($rpoS^+$) and CM1001 (*rpoS**), respectively. Δ*ara* derivatives of CM1000 and CM1001 were made by P1 transduction of Δ*ara714 leu*::Tn*10* from LMG194 (18), selecting for Tc^r; Leu^+ versions were generated by P1 transduction from EM1448 with selection on M63 glucose minimal medium.

The *rne*(Ts) mutation, *rne-3071* (9), was introduced by P1 transduction from EM1277, selecting for a linked marker (*zce*-726::Tn*10*) (31).

The *rnc* gene was deleted and replaced with a *cat* cassette by linear recombination (63). The *cat* cassette from NC397 was amplified by PCR with the primers Rnccatfor and Rnccatrev and electroporated into NM300, selecting for Cm^r, to make CM1223. The entire *rnc* open reading frame from the start codon to the stop codon was deleted in this strain. The start codon of the downstream gene, *era*, overlaps the stop codon of *rnc*, and the start codon of *era* was deleted in this construct. This *rnc* allele will be referred to as Δ*rnc-1223*::*cat*. Despite the fact that *era* is reported to be essential (53), chloramphenicol-resistant colonies were obtained, perhaps through a fusion of the *cat* open reading frame to *era* or readthrough from the *cat* transcript. We confirmed by complementation experiments that the phenotypes studied here were due to the absence of RNase III and not a lack of Era. CM1223 grows slowly at low temperature, similar to other *rnc* mutants (53), and this slow-growth phenotype was complemented by pACS21, a plasmid expressing *rnc* but not *era* (53). Other phenotypes of the Δ*rnc-1223*::*cat* mutant strain (effects on RpoS levels; see Results) were also complemented by this plasmid (data not shown).

Plasmids pNM12, pNM13, pNM33, pNM100, and pNM109 (28, 29) were used to transform appropriate strains and selected with ampicillin, as described previously (10).

Immunodetection of RpoS. Overnight cultures were prepared in LB medium with Ap (100 μg/ml) (LB Ap) at 32°C. Strains were diluted 1:1,000 and grown to mid-exponential phase at 30°C in LB Ap. sRNA expression was induced from plasmids with 0.02% arabinose (KD Medical) for 20 min. One-milliliter samples were taken after the induction, and total protein was collected by trichloroacetic acid (TCA) precipitation, as described previously (42). When the effects of the *rne-3071* allele were tested, cells were heat shocked for 10 min at 43.5°C following sRNA induction, and samples were collected and TCA precipitated immediately before and after heat shock.

Protein samples from equal cell numbers, as determined by measurement of the optical density, were separated by SDS-PAGE using 10% NuPAGE polyacrylamide gels (Invitrogen) and 1× morpholinepropanesulfonic acid (MOPS) buffer, as recommended by the manufacturer. Protein was transferred to nitrocellulose membranes, and RpoS was detected with anti-RpoS antibody (provided by S. Wickner, NIH), as described previously (42). Sample dilutions were included on every gel, and several film (Kodak) exposures were taken to ensure detection in the linear range. Films were scanned with an Epson flatbed scanner at 600 dpi with a calibrated optical density step wedge (Stouffer). ImageJ software was used to quantify the RpoS levels on different exposures, as follows. Each film exposure was calibrated with the step wedge to allow measurement of the optical density. The mean optical density of each band was measured and used for comparison to the mean optical densities of the other bands from the same gel within the linear range. Membranes were also probed with anti-EF-Tu antibody (provided by M. Maurizi, NIH) and analyzed as described above to ensure equal loading of protein on each gel.

RNA preparation and Northern blotting. Overnight cultures were prepared in LB Ap at 32°C. Strains were diluted 1:1,000 and grown to mid-exponential phase at 30°C in LB Ap. sRNA expression was induced from plasmids with 0.02% arabinose for 15 or 20 min, as noted in the legends to Fig. 2 to 6. When mRNA accumulation was measured, samples were collected after the induction for RNA preparation using the hot phenol method, as described previously (31). When the effects of the *rne-3071* allele were measured following the 20-min sRNA induction, cells were heat shocked for 10 min at 43.5°C. Samples were collected immediately before and after the heat shock. To determine mRNA half-lives, rifampin (250 μg/ml) was added after the arabinose induction and RNA samples were collected immediately following addition (time zero samples) and then every 2.5 or 5 min for 20 min, as noted in the legends to Fig. 4. Samples were treated with hot phenol immediately after collection, as described above.

rpoS mRNA was detected by Northern blotting, as follows. Ten micrograms of RNA from each sample was separated by 1.2% agarose gels in 1× MOPS buffer at 100 V. Serial dilutions of at least one sample were included on each gel. RNA was transferred to nylon Nytran N 0.45-μm-pore-size membranes (Whatman) or nylon Zeta-probe membranes (Bio-Rad) using the capillary transfer method in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (Invitrogen) overnight. After UV cross-linking, membranes were incubated with Ultrahyb solution (Ambion) at 42°C for 30 min and then

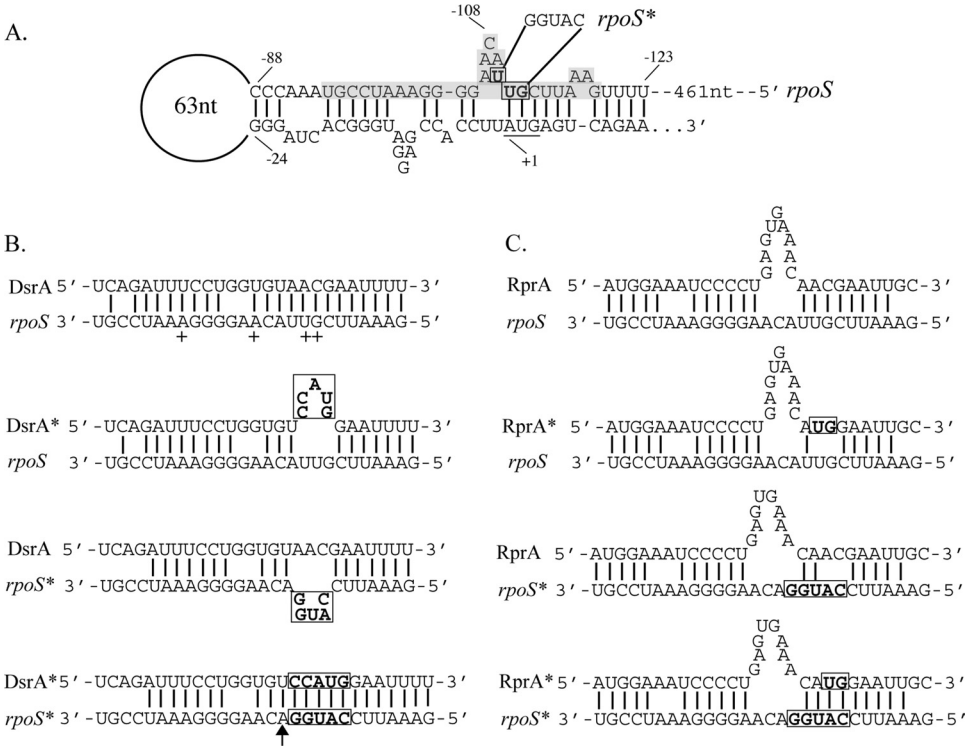

FIG. 1. (A) Nucleotides in the 5′ UTR of the *rpoS* mRNA upstream of the start codon form a hairpin structure in the absence of binding to an sRNA. Bases are numbered backward from the start codon. The transcription start site was mapped at 584 bases upstream of the start codon (see text). The region that pairs with the sRNAs is highlighted, and the start codon is underlined. Mutated bases are boxed, and nucleotide changes in the *rpoS** allele are indicated. (B) Predicted pairings are shown between the *rpoS* or *rpoS** mRNA with DsrA or DsrA*. The plus sign and the arrow indicate 5′ ends of the cleaved forms of *rpoS* mRNA as mapped by 5′ RACE (+, high-temperature forms; arrow, DsrA*-*rpoS** forms). Boxed nucleotides indicate the bases that were mutated in the *dsrA** and *rpoS** alleles. (C) Predicted pairings between the *rpoS* or *rpoS** mRNA with RprA or RprA* are shown, and boxes indicate mutated nucleotides.

hybridized to the RpoS-N3 5′-biotinylated probe (Table 2) (100 ng/ml in Ultrahyb) for approximately 15 to 20 h at 42°C. RpoS-N3 anneals to the *rpoS* mRNA 93 nucleotides downstream of the start codon. The blots were washed, and labeled RNA was detected using a BrightStar BioDetect kit (Ambion), as recommended by the manufacturer. RNA levels in each sample were measured after exposure to film (Kodak) using ImageJ, as described above for the RpoS immunoblots. After exposure to film, the blots were stripped of probe by incubation in boiling 0.5% SDS for 10 min, washed three times in diethyl pyrocarbonate (DEPC)-treated water, and then hybridized as described above with a 5′-biotinylated probe for SsrA (17). Detection and analysis of SsrA levels were as described above. The *rpoS* mRNA accumulation reported for a given sample was normalized to the SsrA level in the same sample; very little variation in SsrA was observed.

5′ Rapid amplification of cDNA ends (RACE) of *rpoS* mRNA. RNA samples were prepared from CM1001/pNM33 following induction with arabinose or from CM1010/pNM13 following induction with arabinose and heat shock at 43.5°C, using hot phenol as described above. Ten micrograms of each RNA sample was treated with 4 U Turbo DNase (Ambion) for 30 min at 37°C. Following phenol-chloroform extraction and ethanol-sodium acetate precipitation, samples were split and half was treated with tobacco acid pyrophosphatase (Epicentre), as described previously (1), while the other half was left untreated. Ligation of the 5′ ends to an RNA adapter was performed as described previously (1). cDNA was obtained by reverse transcription with the SuperScript III enzyme (Invitrogen) and the primer rpoSRACE235, as recommended by the manufacturer. cDNA products were amplified by PCR with an adapter-specific primer and the gene-specific primer rpoSRACE235. PCR products were separated on a Tris-acetate-EDTA agarose gel, purified with a kit (Qiagen), and cloned using the TOPO-TA cloning kit (Invitrogen), as recommended by the manufacturers. Several clones were sequenced with the rpoSRACE235 primer.

RESULTS

One of the best-studied examples of sRNA-mediated positive regulation is provided by the regulation of *rpoS* by DsrA and RprA. Base pairing between the sRNA and *rpoS* mRNA (Fig. 1) is absolutely required for efficient RpoS translation. Specific point mutations in the sRNA or in *rpoS* that disrupt this pairing prevented stimulation of an *rpoS-lacZ* fusion by DsrA or RprA (28, 29). Restoration of pairing through compensatory mutations restored stimulation of the fusion. We have built on these observations to investigate in more detail the mechanism and consequences of positive regulation by these small RNAs *in vivo*.

Previous work examining the mode of action of DsrA and RprA used translational fusions to *lacZ*. In order to look at the roles of DsrA and RprA in the context of the wild-type *rpoS* message and still use the required pairing as a way to ensure specificity of action, a mutant derivative of the native *rpoS* gene with a mutation in the region of the 5′ leader necessary for pairing with DsrA or RprA was created (Fig. 1, *rpoS**). The *rpoS** chromosomal mutation was sufficient to block activation of translation of *rpoS**-*lacZ* by DsrA and limited activation of translation by RprA. The mutant forms of the sRNAs, *dsrA** and *rprA**, restored pairing to *rpoS** (Fig. 1) and activation of the fusion (28, 29).

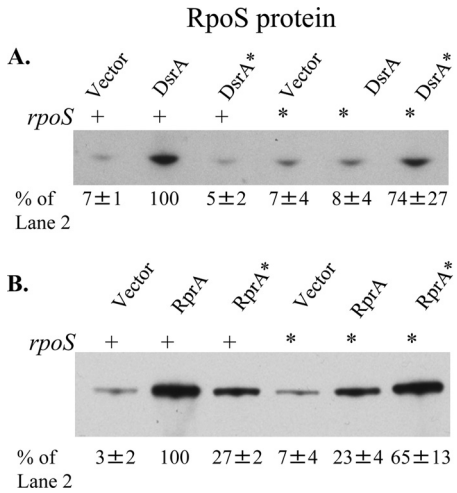

FIG. 2. RpoS expression in strains with optimal or mismatched pairing between sRNAs and *rpoS*. CM1062 (Δ*dsrA rprA*::*kan rpoS*$^+$ Δ*ara714*) and CM1063 (Δ*dsrA rprA*::kan *rpoS** Δ*ara714*) with the pNM12 vector or its derivatives expressing *dsrA* or *dsrA** (A) and CM1062 and CM1063 with the vector or its derivatives expressing *rprA* or *rprA** (B) were grown in LB Ap at 30°C to mid-exponential phase, and sRNA expression was induced with 0.02% arabinose for 20 min. Total protein was precipitated and analyzed by Western blotting. RpoS accumulation in each strain is described as a percentage of the accumulation in the *rpoS*$^+$/pBAD-*dsrA*$^+$ (A) or *rpoS*$^+$/pBAD-*rprA*$^+$ (B) strain. Accumulation is presented as the mean percentage ± the standard deviation ($n = 3$).

RpoS protein levels were measured in these backgrounds by immunoblotting to confirm the effectiveness of sRNA pairing with the wild-type and mutant *rpoS* (Fig. 2). For this analysis, *dsrA rprA* double mutant strains with a wild-type copy of *rpoS* or the *rpoS** mutation were transformed with vector or plasmids containing *dsrA*, *rprA*, *dsrA**, or *rprA** under the control of the arabinose-inducible P_{BAD} promoter (see base-pairing combinations in Fig. 1).

As was observed with the translational fusions, there was little or no detectable RpoS when only the vector was present. However, as with the *rpoS-lacZ* fusion (28), wild-type DsrA significantly stimulated RpoS accumulation (14-fold in Fig. 2A; in other experiments, even higher fold changes were seen [see Fig. 5B]). Expression of DsrA from the P_{BAD} promoter under these conditions yielded 3-fold more RpoS than the amount from strains expressing DsrA from the chromosomal gene under these growth conditions (data not shown). In contrast, the DsrA* derivative, which is unable to fully pair, had no stimulatory effect (Fig. 2A). Mutating *rpoS* to reduce pairing (*rpoS**) also eliminated most RpoS synthesis in the presence of DsrA. As with the translational fusions (28), expressing the *dsrA** plasmid in a host carrying the *rpoS** allele restored RpoS accumulation (Fig. 2A), though the RpoS levels were reduced compared with those from the wild-type pairing. The level of RpoS accumulation in the strains with the paired compensatory mutations was generally somewhat lower than that of the strains with the wild-type combination, suggesting that this region of the 5′ UTR may have some direct effects beyond its role in pairing.

The results with expression of RprA are consistent with this picture. RprA expressed from the plasmid stimulated RpoS translation significantly. In the case of the RprA mismatched sets, however, the *rprA**-*rpoS*$^+$ and *rprA*$^+$-*rpoS** combinations gave significant amounts of RpoS, although they were less than those in cells in which *rprA* and *rpoS* have either the wild type or *rprA**-*rpoS** pairing (23 to 27% of the amount for *rprA*$^+$-*rpoS*$^+$) (Fig. 2B). This finding is similar to results seen with these mutations when monitored with *rpoS-lacZ* fusions (29). The level of RpoS accumulation in the strains with the paired compensatory mutations was 65% that of the wild type.

Stability of *rpoS* mRNA. The *rpoS* mRNA was examined in the presence and absence of optimal pairing with the sRNAs. RNA isolation was performed on a *dsrA rprA* double mutant transformed with the same plasmids as described above, and samples were analyzed by Northern blotting using the oligonucleotide probe RpoS-N3 (Table 2).

When cells expressed neither DsrA nor RprA, *rpoS* mRNA levels were very low (Fig. 3A and B). When wild-type DsrA or RprA can pair with wild-type *rpoS*, the mRNA accumulated to a high level (Fig. 3A and B). The 1.6-kb mRNA detected here is what we expected for a transcript starting at the P_{rpoS} promoter within the *nlpD* open reading frame (23, 52). 5′ RACE analysis of the full-length *rpoS* mRNA identified the same transcription start site mapped previously (23, 52). Quantitative analysis of the Northern blots shows that DsrA or RprA expression from the plasmid resulted in a 7-fold higher level of *rpoS* mRNA compared to that in strains carrying the vector control (Fig. 3A and B). These results suggest that expression of DsrA or RprA stabilized the *rpoS* mRNA. The effect of the sRNAs on the *rpoS* mRNA half-life is addressed below.

To explore the role of base pairing with the sRNAs on *rpoS* mRNA, the mRNA accumulation was examined in strains where sRNA base pairing with the *rpoS* mRNA was disrupted by mutation. In strains expressing DsrA* in the *rpoS*$^+$ background or expressing DsrA in the *rpoS** background, *rpoS* and *rpoS** mRNA levels remained very low (Fig. 3A). This is consistent with a requirement for productive base pairing (leading to translation) of DsrA to *rpoS* mRNA for stabilization of the mRNA.

Cells in which pairing was restored (*dsrA**-*rpoS**) had elevated levels of *rpoS* mRNA (Fig. 3A). Surprisingly, the mRNA accumulating in the *dsrA**-*rpoS** strain was 0.5 kb shorter than the full-length *rpoS* mRNA (Fig. 3A). Northern analysis using a probe that anneals to the 5′ end of *rpoS* mRNA did not detect this truncated band, suggesting that the shorter RNA is missing sequences at the 5′ end upstream of the pairing region (data not shown). Some full-length *rpoS* mRNA can be detected in this background, and some of the truncated form can be detected in the wild type with long exposures (data not shown).

5′ RACE was performed to determine the 5′ end of the truncated form of the *rpoS* mRNA in the *dsrA**-*rpoS** background. This analysis identified an A at the 5′ end, −109 bases upstream of the AUG start codon (marked with an arrow in Fig. 1B), within the region that pairs with DsrA. This result was obtained both from RNA samples treated with tobacco acid pyrophosphatase and from untreated samples, suggesting that this is a cleavage product.

The endoribonuclease RNase III cleaves double-stranded RNA molecules, including mRNAs, in *E. coli* and has been shown to cleave *rpoS* mRNA *in vitro* and to play a role in the

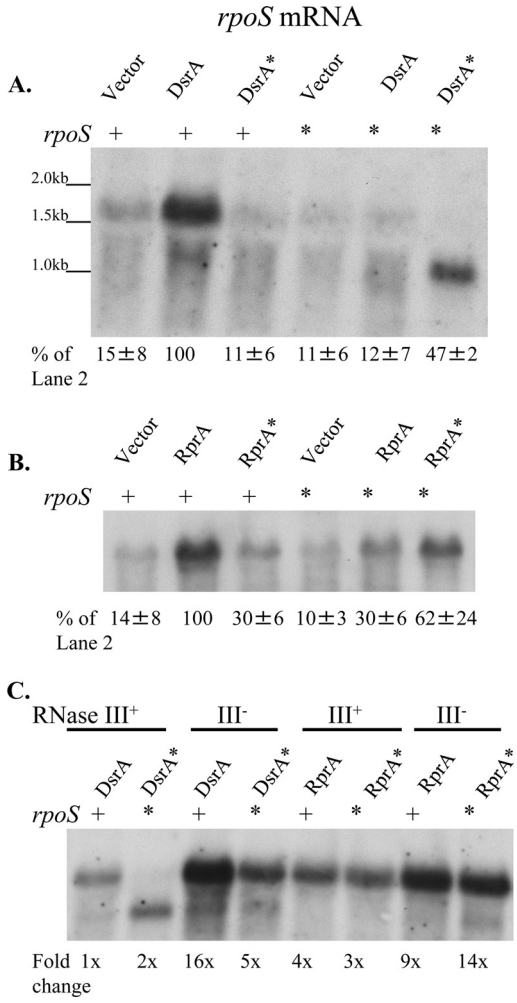

FIG. 3. Northern blot analysis of *rpoS* mRNA levels in strains with optimal or mismatched pairing between sRNAs and *rpoS*. CM1062 (Δ*dsrA rprA*::*kan rpoS*$^+$ Δ*ara*714) and CM1063 (Δ*dsrA rprA*::*kan rpoS** Δ*ara*714) with the pNM12 vector or its derivatives expressing *dsrA* or *dsrA** (A) and CM1062 and CM1063 with the pNM12 vector or its derivatives expressing *rprA* or *rprA** (B) were grown in LB Ap at 30°C to mid-exponential phase, and sRNA expression was induced with 0.02% arabinose for 20 min. RNA was collected by the hot phenol method and analyzed by Northern blotting with the probe RpoS-N3. *rpoS* mRNA accumulation is described as a percentage of the accumulation in the *rpoS*$^+$/pBAD-*dsrA*$^+$ (A) or *rpoS*$^+$/pBAD-*rprA*$^+$ (B) strains. Accumulation is presented as the mean percentage ± the standard deviation (n = 3). (C) *rpoS* mRNA was examined in the same manner as for panels A and B for the RNase III mutant strains (CM1082 and CM1083) containing the same plasmids. *rpoS* mRNA accumulation was measured relative to that of the *rpoS*$^+$ *rnc*$^+$–pBAD-*dsrA*$^+$ strain.

degradation of *rpoS* mRNA and the accumulation of RpoS *in vivo* (14, 44). Northern blot analysis of *rpoS* mRNA accumulation in the *dsrA**-*rpoS** strain in combination with a deletion of *rnc*, the gene encoding RNase III, showed that the mRNA is primarily full length in this background (Fig. 3C). This finding suggests that the cleaved form of *rpoS** seen in the *rnc*$^+$ background is generated by RNase III. Additionally, full-length *rpoS* mRNA levels in the *dsrA*$^+$-*rpoS*$^+$ strain were much higher in the Δ*rnc-1223*::*cat* mutant than in the *rnc*$^+$ background (Fig. 3C). In a strain background expressing *dsrA* from its native promoter on the chromosome, the levels of *rpoS* mRNA were increased 3- to 4-fold in the Δ*rnc-1223*::*cat* mutant relative to those in an isogenic *rnc*$^+$ strain (data not shown). Together, these findings show that RNase III plays a complex role in the cleavage and stability of *rpoS* mRNA, and this role is discussed further in the next section.

In the case of RprA, the effect of disrupting base pairing with the *rpoS* mRNA by the mutations in Fig. 1 was less drastic that that seen for DsrA and *rpoS*, although protein levels were the highest when RprA and *rpoS* had optimal pairing (Fig. 2A and B). Consistent with this, the accumulation of *rpoS* mRNA was also the highest in the cases where pairing was optimal and was reduced but not abolished when pairing was incomplete (Fig. 3B). As with RpoS protein levels, the *rpoS* mRNA levels in the *rprA**-*rpoS** strain were close to, but less than, the levels of *rpoS* mRNA in the strain with wild-type pairing (62%; Fig. 3B). Unlike the *dsrA**-*rpoS** case, however, no truncated *rpoS* mRNA was detected.

Surprisingly, *rpoS* mRNA accumulated to a 3- to 4-fold higher level, on average, with induction of RprA than with DsrA, when the levels of accumulation were compared directly (Fig. 3C). However, *rpoS* mRNA is less susceptible to RNase III in the presence of RprA than it is in the presence of DsrA. The levels of *rpoS* mRNA increased only 2- to 3-fold in the Δ*rnc* background with RprA expression (Fig. 3C). A bulge in the predicted paired sequence of RprA with *rpoS* mRNA near the likely site of RNase III action may make the RprA-*rpoS* duplex a poorer substrate for the enzyme (Fig. 1).

Increased accumulation of *rpoS* mRNA most likely reflects increased stability of the mRNA during the 20-min period of DsrA or RprA expression, since the *rpoS* mRNA levels were low in their absence. To measure the stability of *rpoS* mRNA directly, strains with wild-type *rpoS* or with *rpoS** were induced for expression of DsrA, RprA, DsrA*, or RprA* and treated with rifampin, and RNA was collected at regular intervals. In strains with no DsrA or RprA expression, *rpoS* mRNA levels were too low to measure the half-life (data not shown; note that the chromosomal copy of *dsrA* is also absent from these strains). When DsrA was expressed from the P_{BAD} promoter in the *rpoS*$^+$ strain, the *rpoS* mRNA half-life was 3 min and the truncated form of *rpoS* in the *rpoS**-*dsrA** strain had a similar half-life of 2 min (Fig. 4A). In strains where pairing was disrupted by the mismatch mutations in *dsrA* or in *rpoS*, the half-life could not be measured, because the mRNA became undetectable immediately after the chase began (data not shown). This is consistent with the findings presented in a previous report showing some increase in the half-life of *rpoS* upon overexpression of DsrA, though the authors stated that the half-life was difficult to measure due to the overall instability of *rpoS* mRNA (24). The *rpoS* mRNA half-lives in the *rprA*$^+$-*rpoS*$^+$ and *rprA**-*rpoS** strains were always higher than those in the unpaired sets and higher than the half-lives measured with DsrA expression, although significant variation in these values was seen from experiment to experiment (see Fig. 4 legend). In a representative experiment, the half-life measured for the *rpoS* mRNA in the *rprA*$^+$-*rpoS*$^+$ strain was 10 min, and that for the *rprA**-*rpoS** strain was 15 min (Fig. 4B). In strains where pairing was disrupted (*rprA*$^+$-*rpoS** and *rprA**-*rpoS*$^+$), the half-lives were reduced to 2 min (Fig. 4B). Pairing

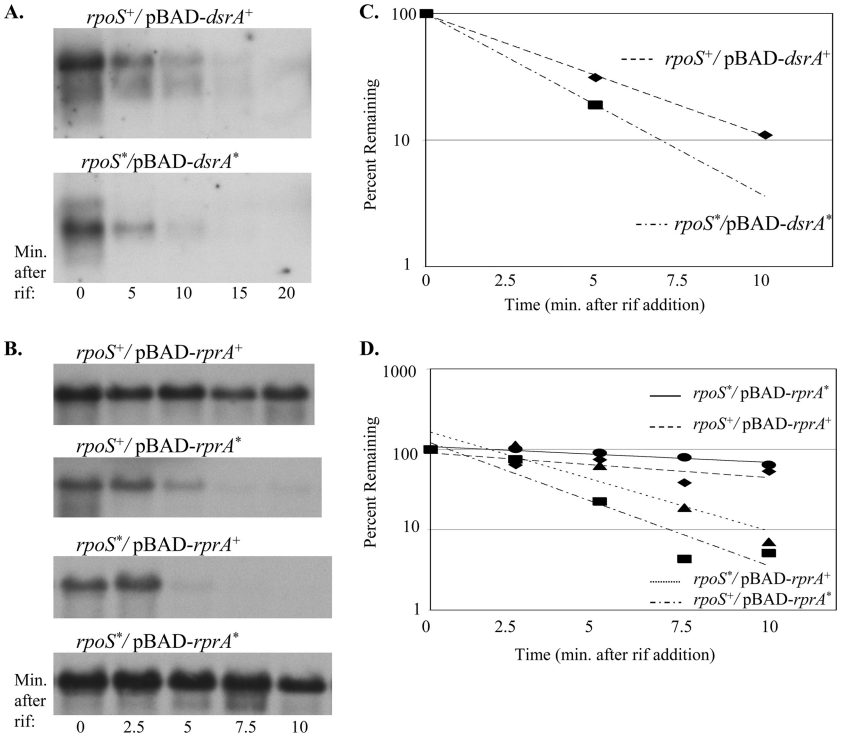

FIG. 4. *rpoS* mRNA half-life determination when it is paired with DsrA or RprA. CM1000 (Δ*dsrA rpoS*$^+$)/pBAD-*dsrA* and CM1001 (Δ*dsrA rpoS**)/pBAD-*dsrA** (A) and JNB001 (Δ*dsrA rprA*::*kan rpoS*$^+$) and JNB002 (Δ*dsrA rprA*::*kan rpoS**) with pBAD-*rprA* or pBAD-*rprA** (B) were grown in LB Ap at 30°C to mid-exponential phase, and sRNA expression was induced with 0.02% arabinose for 15 min. RNA samples were collected by the hot phenol method at the indicated times after the addition of 250 μg/ml rifampin (rif). *rpoS* mRNA levels were analyzed by Northern blotting. The experiment whose results are shown in panel A was performed three times, resulting in half-lives measured for *rpoS* and *rpoS** of 2 to 3 min in each case. The experiment whose results are shown in panel B was performed four times, with some variability in half-lives being measured for *rpoS* and *rpoS**. In each experiment, the mRNA half-lives of the mismatched pairs (*rpoS*$^+$-*rprA** and *rpoS**-*rprA*$^+$) are shorter than that of the optimally paired sets. However, the mRNA half-lives in the *rpoS*$^+$-*rprA*$^+$ and *rpoS**-*rprA** strains varied from 4 to 15 min, and the mRNA half-lives in the mismatched pairs varied from 1 to 10 min. (C and D) Graphical representation of *rpoS* mRNA decay.

between RprA and *rpoS** or RprA* and *rpoS* is apparently sufficient to allow some partial stabilization of the mRNA, as well as to partially allow translation, as seen in Fig. 2B.

When cells with wild-type chromosomal *dsrA* were grown at 25°C (a permissive temperature for DsrA expression) to mid-exponential phase and treated with rifampin, *rpoS* mRNA had a half-life of approximately 2 to 4 min (data not shown), comparable to that seen with DsrA overexpression at 30°C (Fig. 4A). In a Δ*dsrA* strain, *rpoS* decay was too fast to measure; *rpoS* could be detected in the time zero sample only.

These results demonstrate that DsrA and RprA pairing to *rpoS* mRNA not only increases protein translation, as shown above, but also increases mRNA stability and that disruption of base pairing is sufficient to destabilize the mRNA.

Degradation of *rpoS* mRNA by RNase III- and RNase E-mediated mechanisms. The results described thus far show that *rpoS* mRNA is stabilized by base pairing with DsrA and RprA. Thus, in the absence of pairing, the *rpoS* mRNA must be degraded by one or more RNases. RNase E and RNase III are two major RNA endonucleases in *E. coli*; both have been implicated in initiating degradation of sRNAs and mRNAs (20, 31, 35, 61). Cleavage of double-stranded mRNA regions by RNase III can lead to mRNA decay but can also lead to activation of mRNA translation (reviewed in reference 13). In fact, RNase III has been shown to play a role in the degradation of *rpoS* mRNA and the accumulation of RpoS *in vivo* (14, 44). Above, we showed that RNase III can generate a stable cleavage product in the *dsrA**-*rpoS** background. RNase E, in contrast, cleaves single-stranded RNA in AU-rich regions and acts in concert with the degradosome proteins, including the exoribonuclease PNPase, to degrade mRNA (reviewed in reference 8).

A set of isogenic strains was constructed to evaluate the roles of both endoribonucleases in the turnover of *rpoS* mRNA in the presence and absence of DsrA, as well as to evaluate the contributions of mRNA instability on the translation of RpoS. The strains used carried a deletion of *dsrA*, with or without a deletion of the gene encoding RNase III, *rnc*, and with a wild-type or a temperature-sensitive (Ts) allele of the gene encoding RNase E, *rne*. All strains carried either the vector or the pBAD-*dsrA*$^+$ plasmid. After these strains were grown to mid-exponential phase at 30°C, DsrA synthesis was induced with arabinose for 20 min and RNA and protein were isolated after the induction. Cultures were then transferred to 43.5°C for a 10-min heat shock to inactivate RNase E, and RNA and protein samples were again collected. *rpoS* mRNA was analyzed by Northern blotting, and RpoS protein levels were measured by Western blotting analysis. mRNA and protein levels were normalized to those for the wild-type strain (*rnc*$^+$ *rne*$^+$ carrying the vector control).

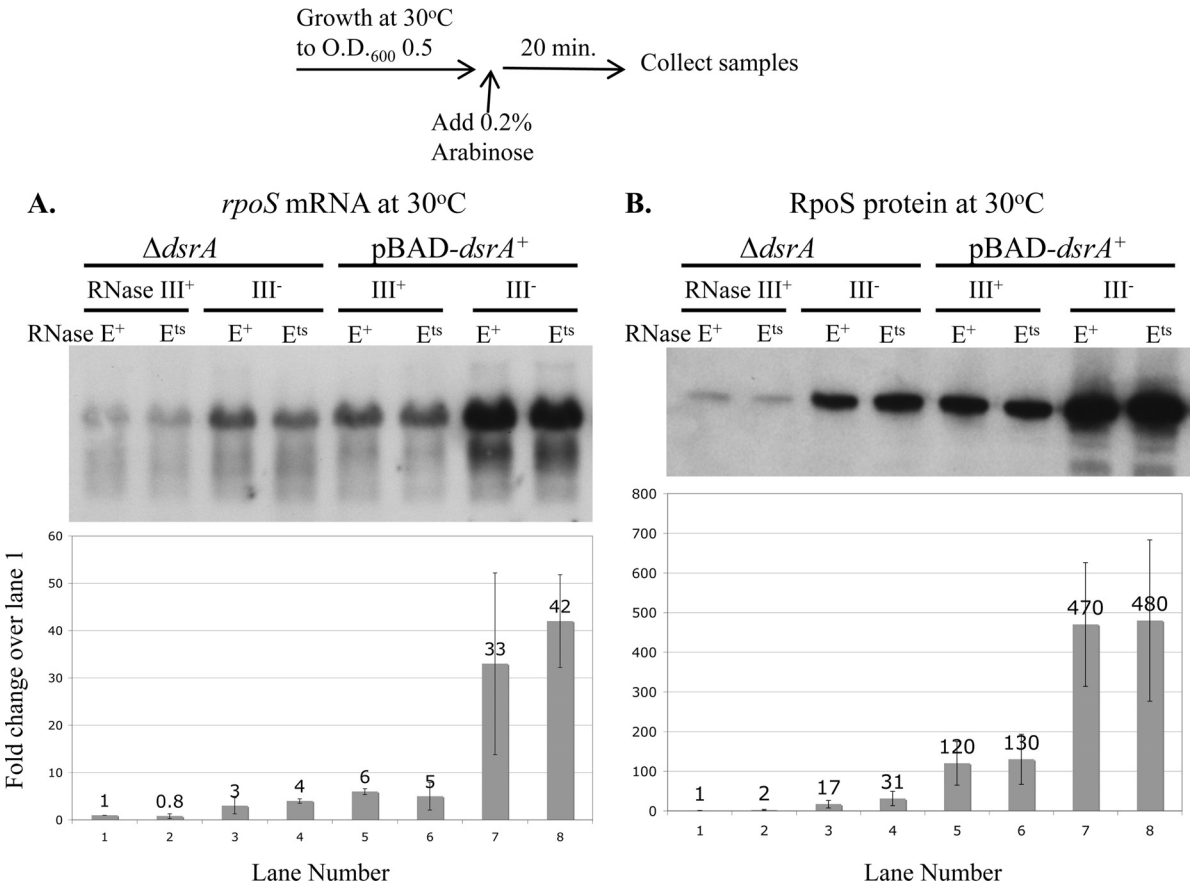

FIG. 5. *rpoS* mRNA and RpoS levels in a Δ*rnc* mutant strain in the presence and absence of DsrA. CM1000 (*rnc*$^+$ *rne* wild type), CM1010 [*rnc*$^+$ *rne*(Ts)], CM1050 (Δ*rnc*-1223::*cat rne* wild type), and CM1052 [Δ*rnc-1223*::*cat rne*(Ts)] with the pNM12 vector or pBAD-*dsrA* were grown at 30°C in LB Ap to mid-exponential phase; sRNA expression was induced with 0.02% arabinose for 20 min. Samples were collected for protein and RNA isolation, as described in Materials and Methods ($O.D._{600}$, optical density at 600 nm). (A) Northern blot analysis of RNA samples was performed using the oligonucleotide probe RpoS-N3 to detect *rpoS* mRNA. (B) Western blot analysis of protein samples was performed using the anti-RpoS antibody to detect RpoS. Graphical analyses show the mean accumulation of the full-length *rpoS* mRNA or RpoS protein ± the standard deviation ($n = 3$) relative to that of CM1000 with the vector control. Samples were also tested by Western blotting for EF-Tu, which was close to identical in each sample. Values over the bars indicate the mean fold change.

Figure 5 shows *rpoS* mRNA and RpoS protein levels after induction of DsrA synthesis at 30°C. In these samples, RNase E should have been active in both the *rne*$^+$ and *rne*(Ts) strains. Therefore, these two alleles had similar phenotypes under this condition. Consistent with the results shown above (Fig. 2 and 3), the *rpoS* mRNA and RpoS protein levels were low in the absence of *dsrA* (Fig. 5A and B). In the Δ*rnc* and Δ*dsrA* background, there was an increase in the *rpoS* mRNA level (3-fold; Fig. 5A) and the RpoS protein level (17-fold; Fig. 5B) compared to the levels in the *rnc*$^+$ Δ*dsrA* strain. Therefore, RNase III plays a role in degradation of the *rpoS* mRNA, as shown by Resch et al. (44), and negatively regulates RpoS translation in the absence of DsrA.

As seen earlier (Fig. 2 and 3), expression of DsrA led to an increase in *rpoS* mRNA levels (6-fold; Fig. 5A) and a significant induction of RpoS translation (120-fold; Fig. 5B) in the *rnc*$^+$ strains. *rpoS* mRNA levels were higher in Δ*rnc* strains than in *rnc*$^+$ strains in the presence of DsrA (6-fold), and there is a 4-fold increase in translation (Fig. 5A and B). Notably, expression of DsrA in the Δ*rnc* background increased both the *rpoS* mRNA level and the level of translation of RpoS significantly, comparable to the increase seen in the *rnc*$^+$ host (Fig. 5A and B). Therefore, RNase III decreases accumulation of *rpoS* mRNA and translation of RpoS significantly in both the absence and presence of DsrA. Furthermore, DsrA works to stimulate translation with or without RNase III.

In the Δ*rnc* cells expressing DsrA (and having high levels of *rpoS* mRNA), a short form of the *rpoS* mRNA was detected (Fig. 5A), suggesting that there could be cleavage by another enzyme. Truncation of the *rpoS* mRNA is discussed further below.

Figure 6 shows *rpoS* mRNA levels and RpoS protein levels after DsrA induction followed by a 10-min heat shock. The 10-min heat shock treatment itself increased the basal level of expression of RpoS protein in the *rne* wild-type background in the presence or absence of DsrA by as much as 10- to 20-fold compared to the levels shown in Fig. 5 (data not shown; see Fig. 6 legend for further detail). This is consistent with the findings of previous studies showing increased RpoS after heat shock (21, 36). While Muffler et al. reported that this increase in RpoS was primarily due to stabilization of the protein (36), we also see a 3-fold increase in the levels of *rpoS* mRNA after

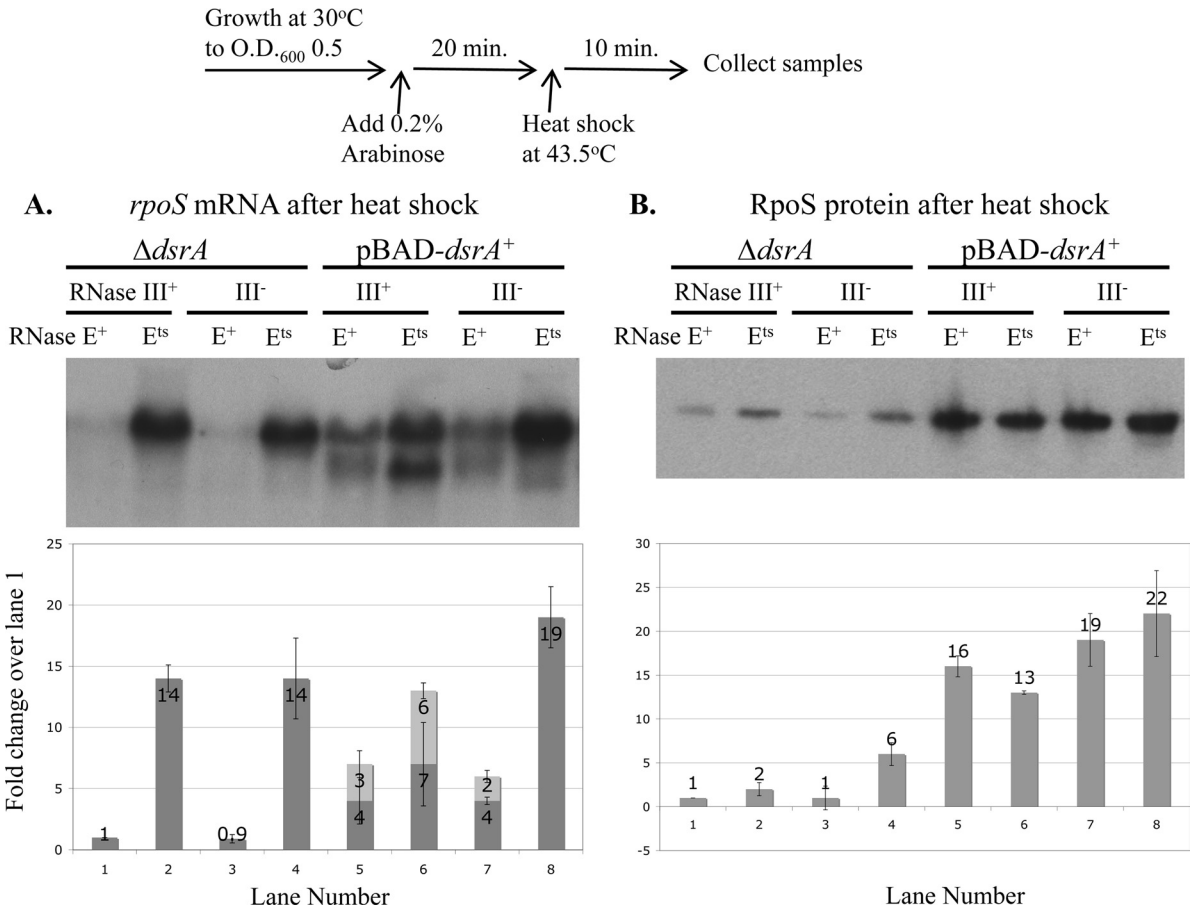

FIG. 6. *rpoS* mRNA and RpoS levels in an *rne*(Ts) mutant strain in the presence and absence of DsrA. CM1000 (rnc^+ *rne* wild type), CM1010 [rnc^+ *rne*(Ts)], CM1050 (Δ*rnc rne* wild type) and CM1052 [Δ*rnc rne*(Ts)] with the pNM12 vector or pBAD-*dsrA* were grown at 30°C in LB Ap to mid-exponential phase; sRNA expression was induced with 0.02% arabinose for 20 min. Cultures were heat shocked for 10 min at 43.5°C, and RNA and protein samples were collected, as described in Materials and Methods ($O.D._{600}$, optical density at 600 nm). (A) Northern blot analysis of RNA samples was performed using the oligonucleotide probe RpoS-N3 to detect *rpoS* mRNA. Graphical analysis shows the mean accumulation of full-length *rpoS* mRNA (dark bars) and the truncated form of the *rpoS* mRNA (light bars) ± the standard deviation (n = 3) relative to that of CM1000 with the vector control. *rpoS* mRNA accumulation in lane 1 is 3 times that for the same strain before heat shock (at 30°C). (B) Western blot analysis of protein samples was performed using the anti-RpoS antibody to detect RpoS. Protein samples were diluted 5-fold before electrophoresis. Graphical analysis shows mean accumulation of RpoS protein ± the standard deviation (n = 3) relative to that of CM1000 with the vector control. RpoS protein accumulation in lane 1 is 20 times that of the same strain before heat shock; in lane 5, RpoS accumulation is 10 times that of the same strain before heat shock. As with Fig. 5, equal protein input per lane was confirmed by Western blotting for EF-Tu. Values over the bars indicate the mean fold change.

the heat shock (data not shown); the effects described here are relative to these increased basal levels.

As described above, in the absence of DsrA, *rpoS* mRNA levels were low compared to those of other samples in this set, as was the RpoS protein level (Fig. 6A and B). Under these conditions, inactivation of RNase E resulted in a significant accumulation of full-length *rpoS* mRNA (14-fold; Fig. 6A), suggesting that RNase E plays a major role in degradation of *rpoS* in the absence of DsrA. However, there was only a 2-fold accumulation of RpoS protein (Fig. 6B, lane 2). Therefore, in the absence of DsrA, inactivation of RNase E is not sufficient for efficient translation of RpoS.

There was little or no effect of the *rnc* mutation on either *rpoS* mRNA or RpoS protein after the heat shock (Fig. 6A and B). However, there was a slightly larger increase in RpoS production when RNase E was inactivated in the absence of RNase III (Fig. 6B, lane 4), which may reflect increased accumulation of RpoS in the *rnc* mutant strain during growth before the heat shock.

DsrA expression resulted in an increase in the total *rpoS* mRNA level (7-fold) and the level of induction of RpoS translation (16-fold) compared to the levels for the vector controls in a Δ*dsrA* host (Fig. 6A and B). Interestingly, however, 40% of the *rpoS* mRNA was truncated in the presence of DsrA (see below). Inactivation of RNase E resulted in only a 2-fold additional increase in the levels of both the full-length and short forms of *rpoS* mRNA and no increase in the level of RpoS (Fig. 6A and B). These data suggest that DsrA expression overcomes most but not all of the RNase E-mediated degradation of *rpoS* and can induce maximal translation of RpoS even in the presence of RNase E. While expression of DsrA stimulated translation in the *rne*(Ts) strain, the level of total *rpoS* mRNA in the pBAD-$dsrA^+$–*rne*(Ts) strain was not any more than that in the pBAD vector-*rne*(Ts) strain, suggesting that DsrA can-

not stabilize the mRNA any further in the *rne*(Ts) strain (Fig. 6A and B, compare lanes 2 and 6).

The truncated form of the *rpoS* mRNA from the *dsrA*$^+$ *rne*(Ts) sample shown in Fig. 6A, lane 6, possesses 5′ ends located in the DsrA pairing region and ranging from −101 to −112 bases upstream of the AUG, as determined by 5′ RACE (Fig. 1B, marked with + signs). Northern blot analysis confirmed that the 5′ end of *rpoS*, upstream of the DsrA interaction region, is missing in this form (data not shown). The short form seen here is the same size as that described above in the *dsrA***-rpoS** strain.

Again, there was no effect of RNase III in the presence of DsrA at this temperature (Fig, 6A and B). However, in the Δ*rnc rne*(Ts) strain there was maximal accumulation of full-length *rpoS* mRNA but little to no accumulation of truncated form (Fig. 6A, lane 8). Cleavage of *rpoS* mRNA in the *rne*(Ts) background requires DsrA and RNase III. Thus, in the Δ*rnc rne*$^+$ strain, some cleavage is seen but must be mediated by an unidentified enzyme.

Taken together, Fig. 6A suggests that RNase E plays an important role in the degradation of *rpoS* mRNA. *rpoS* mRNA is degraded by an RNase E-mediated mechanism in the absence of *dsrA*. DsrA expression stabilizes the *rpoS* mRNA, and DsrA-bound *rpoS* is significantly less susceptible to RNase E. RNase III is not required for RNase E-mediated degradation of *rpoS* mRNA in the absence of *dsrA* or for the DsrA-mediated protection of the *rpoS* mRNA.

A striking effect seen here is that stabilizing *rpoS* mRNA by inactivation of RNase E does not lead to much improvement in translation in the absence of DsrA. DsrA is still required for maximal translation even after the 10-min heat shock and inactivation of RNase E. Thus, although expression of DsrA protects the mRNA from degradation, these data clearly show that protecting from degradation is not sufficient to lead to translation. DsrA plays a separate and critical role by enhancing translation, even under conditions when degradation is significantly reduced.

DISCUSSION

RpoS accumulation is regulated at multiple levels, including the tight regulation of translation dependent upon the RNA chaperone Hfq and the regulatory sRNAs DsrA and RprA. A basic model for stimulation of translation by DsrA and RprA was developed on the basis of predicted base pairing between these RNAs and the upstream leader of *rpoS* mRNA and was confirmed by the demonstration that this base pairing was necessary for sRNA action, using *rpoS-lacZ* translational fusions (28, 29), as well as *in vitro* experiments (26, 49, 58). We looked in more detail at the *in vivo* characteristics of stimulation of RpoS translation by DsrA and RprA, examining the action of these sRNAs on the native *rpoS* mRNA and the translation of RpoS from this mRNA. The advantage of this system is that any characteristics of the mRNA and its degradation are studied under the control of the native promoter and without the use of reporters that may introduce or abolish possible sites of action of ribonucleases or other factors. However, we note that the behavior of the fusions is entirely consistent with what we have observed here.

Previous studies on the action of DsrA and RprA have clearly demonstrated an increase in translation that is dependent on sRNA binding. In *in vitro* experiments, sRNA binding results in remodeling of the *rpoS* mRNA to uncover the ribosome binding site, the likely explanation for the increased translation (26, 58). Our results are entirely consistent with those findings. Our work also shows that overexpression of both DsrA and RprA significantly increases the *rpoS* mRNA half-life and, therefore, the level of mRNA accumulation during the short induction period. The levels of mRNA generally correlate well with the levels of translated protein in these experiments.

One interpretation of this increase in mRNA stability is that it is secondary to increased translation, since it is known that translation helps to protect mRNA from degradation (reviewed in reference 11). An alternative interpretation would be that the sRNAs, by increasing the stability of the mRNA, lead to increased translation. If this were the case, stabilizing the mRNA by inactivating the RNases responsible for degradation should be as effective as the sRNAs in increasing translation. Our results using RNase mutants make the second possibility unlikely.

RNase E and the degradation and translation of *rpoS* mRNA. RNase E is an important and essential endoribonuclease in *E. coli*. It cleaves single-stranded RNA and has previously been implicated in the negative regulation of mRNAs by small RNAs (31, 35, 39). The data presented here suggest a central role for RNase E in the degradation of *rpoS* mRNA. Levels of the mRNA increased significantly when RNase E was inactivated by heat shock in strains with a temperature-sensitive allele of *rne*, the gene encoding RNase E (Fig. 6A). DsrA expression led to very little additional accumulation of RNA when RNase E was inactivated and DsrA was expressed (compare lanes 6 and 8 to lanes 2 and 4 in Fig. 6A). This suggests that DsrA's major effect on *rpoS* mRNA accumulation is in overcoming RNase E-dependent degradation of this mRNA.

These experiments also very clearly show that stabilization of the mRNA is not sufficient to lead to RpoS translation and that the effect of DsrA on mRNA stability is not as strong as the effect on translation. Thus, DsrA played an important role in promoting translation even when *rpoS* mRNA levels were relatively high (Fig. 6B; compare RpoS protein levels in lanes 6 and 8 to those in lanes 2 and 4), and, even more strikingly, protein levels were high when DsrA was expressed, even when not as much mRNA accumulated (Fig. 6B, lanes 5 and 7). Another way to measure the relative effect of DsrA on RpoS translation versus the effect on mRNA stability is by a measure of translation efficiency (a comparison of relative protein levels to relative mRNA levels) (Table 3). If we normalize translation efficiency to that for the wild-type strain in the absence of DsrA at 30°C (set equal to 1), DsrA stimulated translation relative to mRNA levels greater than 20-fold [Table 3, *rnc*$^+$ *rne*$^+$ *dsrA*$^+$ and *rnc*$^+$ *rne*(Ts) *dsrA*$^+$]. At 43.5°C, both protein and mRNA levels were higher, so we separately normalized the translation efficiency to 1 for the wild-type strain in the absence of DsrA under this condition (see below for temperature-specific effects independent of RNase E). At 43.5°C, the stimulation by DsrA was 4-fold in both *rne*$^+$ strains (Table 3, *rnc*$^+$ *rne*$^+$ *dsrA*$^+$ and Δ*rnc rne*$^+$ *dsrA*$^+$) but was only 1-fold when RNase E was inactivated, because the level of RNA was already very high and was barely increased by DsrA (compare lanes 2 and 4 to

TABLE 3. Efficiency of RpoS translation

Strain	Translation efficiency[a]	
	30°C	43.5°C
rnc$^+$ *rne*$^+$ Δ*dsrA*	1	1
rnc$^+$ *rne*(Ts) Δ*dsrA*	2	0.1
Δ*rnc rne*$^+$ Δ*dsrA*	6	2
Δ*rnc rne*(Ts) Δ*dsrA*	8	0.4
rnc$^+$ *rne*$^+$ *dsrA*$^+$	21	4
rnc$^+$ *rne*(Ts) *dsrA*$^+$	24	1
Δ*rnc rne*$^+$ *dsrA*$^+$	14	5
Δ*rnc rne*(Ts) *dsrA*$^+$	12	1

[a] Efficiency of translation of the *rpoS* mRNA was determined as a ratio of the relative RpoS protein levels to the relative *rpoS* mRNA levels normalized to that of the *rnc*$^+$ *rne*$^+$ Δ*dsrA* strain, as measured to obtain the results shown in Fig. 5 and 6.

lanes 6 and 8 in Fig. 6A). Because the levels of RpoS protein were similar when DsrA was present, with or without RNase E (Fig. 6B, compare lane 5 to lane 6 and lane 7 to lane 8), this also suggests that the level of mRNA is not limiting for DsrA-dependent translation.

In a previous study, Urban and Vogel examined the ability of DsrA and RprA to stimulate the translational fusion *rpoS*::GFP (green fluorescent protein) in cells carrying *rne-701*, defective in the degradosome portion of RNase E, and saw significant stimulation there as well (59). Our results significantly extend those observations and demonstrate that DsrA efficiently overcomes the ability of RNase E to degrade the *rpoS* mRNA and allows very efficient translation, whether or not RNase E is functional. We note that Basineni et al. (2) did not see an effect of inactivating RNase E on *rpoS* turnover. We do not currently have an explanation for this difference, although in their experiments cultures were shifted to 42°C, not 43.5°C, which we find necessary for inactivation of RNase E, and were also treated with hydrogen peroxide before the mRNA half-life was measured. In other work (45), a strain with mutations in *rne*, *pnp*, and *rnb* was examined and reported to have increased levels of RpoS compared to those in wild-type cells, consistent with our findings for *rne* mutants, but the contribution of each of these genes was not determined.

RNase III and degradation and translation of *rpoS* mRNA. In *E. coli*, RNase III is a nonessential double-stranded endoribonuclease. Evidence of a role for RNase III in decay of *rpoS* mRNA has been reported (2, 14, 44). The role of RNase III in the degradation of *rpoS* mRNA is rather different and somewhat more complex than that of RNase E. Consistent with the report by Resch et al. (44), we find that RNase III reduces the level of *rpoS* mRNA, presumably by degrading it, and that this degradation occurs even when DsrA is present (44). mRNA levels were increased 3- to 4-fold in the absence of RNase III in the Δ*dsrA* background, and this increase in *rpoS* mRNA was accompanied by a significant (17-fold) increase in protein levels (Fig. 5A and B). Under the conditions of this experiment (absence of *dsrA* in the chromosome, growth of cells at 30°C), RNase III clearly contributes to keeping the levels of RpoS low. Freire et al. (14) found a negative effect, rather than a positive effect, of an RNase III mutation on *rpoS* mRNA stability and protein levels under carbon starvation conditions. We have not tested our mutants under these conditions, but these results suggest additional complexity for the role of RNase III.

While DsrA clearly suppresses the effects of RNase E, it does not overcome the effects of RNase III. Thus, DsrA is additive with the effects of an *rnc* mutant. The level of mRNA increased 10-fold in the *rnc* mutants when DsrA was expressed, and the level of protein increased more than that, so that the level of mRNA and the level of RpoS protein were the highest when DsrA was present and RNase III was absent (Fig. 5, lanes 7 and 8). This suggests that the ability of RNase III to negatively affect *rpoS* is not overcome by translation or by DsrA annealing. This interpretation is somewhat different from that reached by Resch et al. (44), who suggested that translational activation involves RNase III-dependent processing of the *rpoS* 5′ leader (44). The clear activation that we see by DsrA even in the absence of RNase III demonstrates that RNase III cleavage is not necessary for DsrA to act (compare lanes 7 and 8 to lanes 3 and 4 in Fig. 5B).

How and where does RNase III act on *rpoS* mRNA? One possibility that we cannot currently rule out are indirect effects of an *rnc* mutant. However, this discussion is based on the assumption that RNase III directly acts on *rpoS* mRNA. *In vitro*, Resch et al. (44) find RNase III cleavage of the *rpoS* mRNA 5′ UTR in the absence of sRNA at −15 and −94 nucleotides upstream of the start codon, in the paired region of the hairpin. In the presence of chromosomally encoded DsrA, they find cleavage at these two sites as well as at −112 nucleotides upstream of the AUG in the region of pairing with DsrA (44).

In our experimental setup, we are overproducing DsrA, so that pairing with *rpoS* should be relatively complete. If we assume that essentially all *rpoS* mRNA is paired with DsrA, the negative effect of RNase III on RNA accumulation when DsrA is present (Fig. 5A) is consistent with a model in which RNase III can cleave the DsrA-*rpoS* double-stranded structure after hairpin opening. Direct evidence for such a cleavage *in vivo* was seen in the *rpoS**-DsrA* strain (Fig. 3C), where all of the *rpoS* mRNA was cleaved. The 5′ ends of this cleavage product mapped to the DsrA pairing region of the *rpoS* mRNA, at position −109, a few nucleotides from the −112 position found by Resch et al. *in vitro* (Fig. 1) (44). However, we did not see this processed band in the strain with wild-type DsrA and wild-type *rpoS*; if the cleavage occurs, either it is not very efficient or the resulting cleaved *rpoS* mRNA is rapidly degraded. We suggest that both are occurring. Part of the mRNA is cut and destroyed, reducing the extent of RpoS accumulation. Results shown in Fig. 6 suggest that RNase E is participating in the rapid degradation of the cleaved *rpoS* mRNA; there is evidence of accumulation of the truncated message at high temperature, when RNase E was inactivated (Fig. 6, lane 6). Cleavage of *rpoS* mRNA by RNase III will provide a 5′-monophosphate for the remaining *rpoS* message, a better substrate for subsequent RNase E cleavage downstream (12). The rest of the mRNA may not be cut, even though it is paired with DsrA. The uncut mRNA would then be an efficient substrate for translation. It is possible that the wild-type DsrA-*rpoS* duplex may bind to RNase III less effectively than the DsrA*-*rpoS** duplex or that the wild-type duplex may bind to some other factor that prevents RNase III action.

When RprA is present and paired with the *rpoS* mRNA, a

loop in RprA is found in the region where RNase III is found to cleave the DsrA-*rpoS* duplex (Fig. 1C). The RprA*-*rpoS** duplex also contains this bulge and is not truncated like the *rpoS** mRNA paired with DsrA* (Fig. 3B). While there are RNase III-dependent effects on *rpoS* in the presence of RprA (Fig. 3C), the RprA-*rpoS* duplex may be less susceptible to this endonuclease than the DsrA-*rpoS* duplex. This may explain the increased stability of *rpoS* mRNA after RprA expression in *rnc*$^+$ strains compared to that of *rpoS* mRNA after DsrA expression (Fig. 3C and 4).

Effects of high temperature on RpoS translation and mRNA stability. In order to test the effects of inactivating the essential endonuclease RNase E, it was necessary to carry out one set of analyses after a 10-min heat shock at 43.5°C. This has provided some insight into the effect of high temperature on the regulation of *rpoS*. There was an increase in the level of RpoS protein (in the absence of DsrA) of 20-fold, somewhat higher than the increase of 3- to 5-fold after heat shock reported previously (36). In addition, we detected a 3-fold increase in *rpoS* mRNA levels as well. Thus, in addition to the reported stabilization of RpoS protein at high temperature, it seems likely that either there is increased mRNA stability, possibly from increased translation, or increased transcription.

A striking observation was that RNase III showed little or no effect on *rpoS* mRNA and protein levels after the heat shock (Fig. 6). One model for both this observation and the increased mRNA levels might be that heat shock may lead to melting of the secondary structure within the hairpin, rendering it resistant to RNase III at these sites. Alternatively, RNase III could be either inactive or unavailable (busy with other substrates) under this condition. The heat shock-related increase in *rpoS* mRNA and translation may simply be due to loss of RNase III action on the *rpoS* mRNA.

ACKNOWLEDGMENTS

We thank Gisela Storz, Nicholas De Lay, Kyung Moon, Aurelia Battesti, Pierre Mandin, Daniel Schu, and Paola Milanesio for their comments on the manuscript.

This research was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH, and by a National Research Service Award to C.A.M. (award F32 GM075392, National Institutes of Health).

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